Calcium-Induced Associations of the Caseins: Thermodynamic Linkage of Calcium Binding to Colloidal Stability of Casein Micelles

The caseins occur in milk as colloidal complexes of protein aggregates, calcium, and inorganic phosphate. As determined by electron microscopy, these particles are spherical and have approximately a 650 Å radius (casein micelles). In the absence of calcium, the protein aggregates themselves (submicelles) have been shown to result from mainly hydrophobic interactions. The fractional concentration of stable colloidal casein micelles can be obtained in a calcium caseinate solution by centrifugation at 1500 *g*. Thus, the amount of stable colloid present with varying Ca^{2+} concentrations can be determined and then analyzed by application of equations derived from Wyman's Thermodynamic Linkage Theory. Ca^{2+}-induced colloid stability profiles were obtained experimentally for model micelles consisting of only α_{s1}- (a calcium insoluble casein) and the stabilizing protein κ-casein, eliminating the complications arising from β- and minor casein forms. Two distinct genetic variants α_{s1}-*A* and *B* were used. Analysis of α_{s1}-*A* colloid stability profiles yielded a precipitation (salting-out) constant k_1, as well as colloid stability (salting-in) parameter k_2. No variations of k_1 or k_2 were found with increasing amounts of κ-casein. From the variation of the amount of colloidal casein capable of being stabilized vs. amount of added κ-casein an association constant of 4 L/g could be calculated for the complexation of α_{s1}-*A* and κ-casein. For the α_{s1}-*B* and κ-casein micelles, an additional Ca^{2+}-dependent colloidal destabilization parameter, k_3, was added to the existing k_1 and k_2 parameters in order to fully describe this more complex system. Furthermore, the value of k_3 decreased with increasing concentration of κ-casein. These results were analyzed with respect to the specific deletion which occurs in α_{s1}-casein *A* in order to determine the sites responsible for these Ca^{2+}-induced quaternary structural effects.

1. INTRODUCTION

In 1929 Linderstrom-Lang, as a result of his studies on casein, postulated the colloidal milk protein complex (the casein micelle) to be composed of a mixture of calcium-insoluble proteins stabilized by a calcium-soluble protein. The latter protein would be readily split by the milk-clotting enzyme chymosin, destabilizing the colloid and allowing coagulation to occur. This was one of the first demonstrations of a protease-triggered structural cascade, and the system as a whole functions in all species of mammals as a colloidal calcium transport complex. It is this complex which provides virtually all the calcium and phosphate required by neonates (Farrell and Thompson, 1988).

The individual bovine caseins have been isolated and purified and the α_{s1}-, α_{s2}-, and β-caseins have been shown to be calcium-insoluble, while κ-casein is not only soluble in the presence of calcium ions,

but is also readily split by the enzyme chymosin (for a review, see Farrell and Thompson, 1988). In addition, Waugh *et al.* (1971) demonstrated that complexes of α_{s1}- and κ-casein can be reformed from the isolated fractions as measured by sedimentation velocity experiments. Pepper and Farrell (1982) demonstrated the interaction of α_{s1}- and κ-casein by gel filtration and studied the concentration dependence of the interaction. The complexes, formed by the association of the isolated α_{s1}- and κ-caseins, can in turn aggregate to form model colloids upon addition of Ca^{2+} in 0.01 M imidazole buffer, *p*H 6.7. As viewed by electron microscopy, these model colloids are virtually identical with fresh-milk micelles, except for their somewhat altered size (Schmidt, 1984). The precise mechanism of formation of these stable model casein micelles is as yet uncertain, although several theories have been advanced (Farrell and Thompson, 1988). To understand the forces involved in regenerating stable colloid complexes, the precipitation by calcium ions of selected caseins (salting-out) and their stabilization into colloidal complexes (salting-in) were reinvestigated. To quantitate these observations, data were analyzed with mathematical models (Farrell *et al.*, 1988), which have been developed to take into account the thermodynamic linkage (Wyman, 1964) which is evident among the calcium-bound salted-out and salted-in forms of the individual caseins. This model has now been extended to quantitate interactions between calcium and mixtures of α_{s1}- and κ-caseins and to analyze the stability of reformed colloidal complexes.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Purified caseins were prepared as previously described (Thompson, 1966) by DEAE-cellulose chromatography. Stock $CaCl_2$ solutions were prepared and their concentration checked by Atomic Absorption Analysis. Nomenclature of the genetic variants and abbreviations for casein are as previously described (Eigel *et al.* , 1984).

2.2. Solubility of Caseins

Solubility study of caseins at 37°C was carried out as follows:

1. Dissolve caseins (about 20 mg/ml) in water and adjust *p*H to 7.0, with 0.1 N KOH or NaOH. Equilibrate in water bath at desired temperature for 15 to 20 min.
2. To 2 ml of protein solution (in thick-walled centrifuge tubes), blow in 2 ml of $CaCl_2$ solutions, with or without buffer ±KCl, invert the tube, let stand at desired temperature for 30 min.
3. Centrifuge for 15 min at 43,800 *g* max in an SW 60 Ti rotor at fixed desired temperature in a Model L-8 Beckman ultracentrifuge.
4. Transfer 500 μl of supernatant to a 5 ml volumetric flask, containing 1 ml 1 N sodium citrate plus a few milliliters of water; make up to volume with water. When solubility is determined at 1°C, pipettes must be prechilled to avoid precipitation of protein in the pipette. Read in 1-cm cuvettes at 280 nm. Extinction coefficients, ε (1 cm 1% 280 nm) for α_{s1}-*A* and *B* (10.0) for β-casein (4.7) and for κ-casein (10.5), given by Eigel *et al.*, 1984, were used to quantitate the amount of soluble protein.

2.3. Model α_{s1}-Casein Micelles, Colloidal Stability

Stabilization of α_{s1}-caseins by κ-casein in the presence of Ca^{2+} was performed at 37°C. The procedure was as follows:

1. One milliliter of stock α_{s1}-casein solution (40 mg/ml) was added to a 20 ml conical graduated centrifuge tube. The appropriate amounts of κ-casein were added to a series of tubes to achieve ratios of α_{s1}/κ-of 40:1, 20:1, and 10:1 and the mixtures diluted to 5 ml with water.
2. Solutions were warmed to 37°C and 5 ml of desired calcium chloride-imidazole-HCl buffered solution blown into the solution. The tubes were inverted twice and allowed to stand in a 37°C water bath for 30 min.
3. Tubes were centrifuged at 1500 *g* for 10 min.
4. One milliliter of the supernatant was added to a 5 ml volumetric flask containing 1 mL of 1 N sodium citrate, plus 2 ml water and the mixture diluted with water. Turbid solutions were cleared by the addition of 100 μl of 0.1 M Na_2 EDTA and the supernatant protein concentration determined at 280 nm.

2.4. Sedimentation Analysis

Sedimentation velocity studies were performed on a Beckman Model E analytical untracentrifuge equipped with electronic speed control and photo-

metric scanner. The scanner was interfaced with a Modular Computer Systems Model III computer equipped with a real-time analog-to-digital voltmeter set to take readings at 40 msec intervals. The data collection system resulted in a minimum of 200 points per boundary. Each boundary was analyzed by a fit of an integral of a gaussian, by the Gauss–Newton procedure (Kumosinski *et al.*, 1981). For each experiment, a minimum of 15 scans were used to obtain a linear regression of the log of the distance of the boundary from the center of rotation as a function of time. From the regression coefficient, $s_{20,w}$ was calculated with a precision of ±0.1%. Such an analysis is equivalent to a second-moment calculation for schlieren data, and any existing heterogeneity of the sample can be verified easily by the nonlinearity of the log of the position vs. time plot. An additional benefit of the scanner over the schlieren or fringe optical system is that it provides, by virtue of the high sensitivity of absorbance measurements, the means of working at relatively dilute (<1 mg/ml) protein concentrations.

2.5. Theory and Data Analysis

The caseins of all species display characteristic changes in their colloidal stability as a function of added calcium. Individual calcium-sensitive caseins have known association constants for calcium (Dickson and Perkins, 1971), and in a previous study (Farrell *et al.*, 1988) we demonstrated thermodynamic linkage between the free energy of salt binding and the solubility of the caseinates. Here, we have extended that theory to assume that the observed sequential changes in colloidal stability (e.g., Fig. 5b), which occur with increasing calcium concentration, are also thermodynamically linked to calcium binding. Therefore, we have applied the theory of linked functions as developed by Wyman (1964) to treat these processes. The concept employed is that the observed changes in colloidal stability (solubility) with increased ligand concentration, are thermodynamically linked to ligand binding. In a sense these are not *a priori* derived equations but model equations fit to the data thorough iterative analysis and statistical tests. For example, a system of ligand-induced changes as seen in Fig. 5b, involves an initial salting-out, followed by a partial salting-in (the dip in the data between 0.004 and 0.006 M $CaCl_2$) both of which are followed by colloidal instability at concentrations of $CaCl_2 > 0.015$ M. The simplest model which fits the data relating solubility or stability states (S) to calcium binding is as follows:

$$\begin{array}{ccccc} (S_0) & & (S_1) & & (S_2) \\ p+nx & \overset{k_1}{\rightleftharpoons} & PX_n & +mx \overset{k_2}{\rightleftharpoons} & PX_mX_n \\ & & +qx & & +qx \\ & & \Big\Vert k_3 & & \Big\Vert k_3 \\ & & PX_nX_q & +mx \overset{k_2}{\rightleftharpoons} & PX_mX_nX_q \\ & & (S_4) & & (S_3) \end{array} \tag{1}$$

where p is the unbound protein; x is the free salt; n, m, and q are the apparent number of moles bound to species PX_n, PX_nX_m, and $PX_nX_mX_q$; and S_0, S_1, S_2 are the colloid stabilities (solubilities) of the species indicated. S_4 represents an unstable complex not observed in these experiments. The mathematical relationship representing the fractional contributions of each stable species from the above stoichiometry can be represented as follows:

$$S_{app} = S_0 f(p) + S_1 f(PX_n) + (S_2 - S_1) f(PX_nX_m) + S_3 f(PX_nX_mX_q) \tag{2}$$

where S_{app} is the experimentally observed colloid stability (protein solubility) and $f(i)$'s are the protein fractional component of species i and the S's are the solubilities of each species. For this study S_1 and S_2 will be relative to S_0. Incorporation of the salt-binding equilibrium constants as defined by Eq. (1) into (2) leads to the following:

$$S_{app} = \frac{S_0 p}{p + k_1^n p x^n} + \frac{S_1 k_1^n p x^n}{p + k_1^n p x^n} + \frac{(S_2 - S_1) k_2^m p x^m}{p + k_2^m p x^m + k_3^q p x^q + k_2^m p x^m x^q} + \frac{S_3 k_2^m k_3^q p x^m x^q}{(p + k_2^m p x^m)(p + k_3^q p x^q)} \tag{3}$$

where p is the concentration in percent of the unbound protein and x is the concentration of unbound salt. Cancelation of common terms yields:

$$S_{app} = \frac{S_0}{1 + k_1^n x^n} + \frac{S_1 k_1^n x^n}{1 + k_1^n x^n} + \frac{(S_2 - S_1) k_2^m x^m}{1 + k_2^m x^m + k_3^q x^q + k_2^m k_3^q x^m x^q} + \frac{S_3 k_2^m x^m k_3^q x^q}{(1 + k_2^m x^m)(1 + k_3^q x^q)} \tag{4}$$

It should be stressed here that the latter expression represents sequential binding (i.e., $k_1 > k_2 > k_3$), which is a model in accord with the experimentally observed events (of Fig. 5b). Also, for n or m values greater than one, k_1, k_2 and k_3 represent an average value for each of the n, m, or q binding sites. In reality n, m, or q moles of salt will bind with only one equilibrium constant (K_1) (i.e., $K_1 = k_1^n K_2 = k_2^m$ and $K_3 = k_3^q$). Note, however, that the concept of thermodynamic linkage considers only those "binding" events relative to the linked physical change studied.

Now, since the total salt concentration, X_T, is the sum of the free salt concentration, x, and the concentration of the bound salt of all species PX_n, PX_nX_m, and $PX_nX_mX_q$, it can be shown that

$$X_T = x\left(1 + \frac{nk_1^n P_T x^{(n-1)}}{1 + k_1^n x^n} + \frac{mk_2^m P_T x^{(m-1)}}{1 + k_2^m x^m} + \frac{qk_3^q P_T x^{(q-1)}}{1 + k_3^q x^q}\right) \quad (5)$$

where P_T is the total concentration of protein. From Eq. (5) it can be seen that X_T approaches x when P_T is small relative to x. In some of our experiments this assumption is reasonable. For α_{s1}-casein very low (1 to 2 mM) concentrations of calcium induce relatively strong aggregations (Waugh *et al.*, 1971). Dalgleish and coworkers (1981) studied the rates and concentration dependence of the aggregations using a stopped-flow spectrophotometer. They concluded that the kinetically active unit which participates in the precipitation reaction is an octamer, which in turn is in equilibrium with its component monomer. Thus, for colloidal stability experiments conducted at 2 to 4 mg/ml, the kinetically active P_T of Eq. (5) is 2.8×10^{-5} M. In direct protein solubility studies this may not be the case. Using the values of K_a published by Dickson and Perkins (1971) for α_{s1}-casein along with P_T, free x can be calculated and used to generate adjusted k_i's which may be more properly related to an "apparent" binding constant. However, the latter is still an approximation, and from the point of view of the kinetically active species, X_T is also appropriate.

It could be argued that the calcium-induced protein self-associations are a complicating factor in these studies even though the monomer and aggregate are both soluble. Cann and Hinman (1976) developed equations for dealing with the effects of association on ligand binding. Using their concepts, it can be seen that:

$$\begin{array}{ccc} S_0 & \overset{k_0^j}{\rightleftharpoons} & S_0 \\ kp + jx & & P_kX_j + \end{array}$$

$$\begin{array}{ccc} P_kX_j + nx & \overset{k_1^n}{\rightleftharpoons} & P_kX_jX_n \\ S_0 & & S_1 \end{array} \quad (6)$$

and the apparent solubility S_{app} again is equal to the sum of the solubilities of each species times the fraction of the protein in that species.

$$S_{app} = S_0 f_p + S_0(f_{P_kX_j} - f_{P_kX_jX_n}) + S_1 f_{P_kX_jX_n} \quad (7)$$

Thus,

$$S_{app} = S_0 f_p + S_0 f_{P_kX_j} + (S_1 - S_0) f_{P_kX_jX_n} \quad (8)$$

$$S_{app} = S_0\left[\frac{p}{p + k_0^j P^k x_j} + \frac{k_0^j P^k x^j}{p + k_0^j P^k x^j}\right] + (S_1 - S_0)\frac{P_kX_jX_n}{P_xX_j + P_kX_jX_n}$$

and

$$S_{app} = S_0 \frac{k_0^j P^k x^j + p}{p + k_0^j P^k x^j} + (S_1 - S_0)\frac{k_1^n (P_kX_j) x^n}{(P_kX_j) + k_1^n (P_kX_j) x^n} \quad (9)$$

Collection of terms yields:

$$S_{app} = \frac{S_0}{1 + k_1^n x^n} + \frac{S_1 k_1^n x^n}{1 + k_1^n x^2} \quad (10)$$

It can be seen that Eq. (10) is now in the same form as Eq. (4) and that the association parameter k_0^j has canceled out. Thus, only binding sites linked to changes in solubility will be seen in this analysis. This result is in line with the experimental data collected by Dalgleish *et al.* (1981), which also showed that the associated octomer alone participates in the precipitation reaction.

Salt-induced colloid stability (solubility) profiles were directly analyzed using a Gauss–Newton nonlinear regression analysis program developed at this laboratory by Dr. William Damert. All profiles were analyzed by fixing the values of n and m or q and calculating the best least-squares fit for the optimum evaluated k_1, k_2, and k_3 values. The n, m, and q values were then fixed to new integer values and the entire procedure was repeated. The n, m, and q values which yielded the minimum root-mean-square value for the analysis with the minimum error in k_1, k_2, and k_3 were then reported.

The colloid stability of α_{s1}-caseins induced by κ-casein at 37°C can be studied in two ways, the general equation above can yield values for salt-induced changes and the total percent protein soluble at elevated Ca^{2+} can be analyzed for its dependence on κ-casein concentration. To obtain an apparent constant for the interaction:

$$[\alpha_{s1}\text{-}]+[\kappa\text{-}] \overset{K}{\rightleftharpoons} [\alpha_{s1}\text{-}\kappa\text{-}] \qquad (11)$$

Here the final amount solubilized

$$S_i k_i = \frac{C_0}{1+K^n X^n} + \frac{C_1 K^n X^n}{1+K^n X^n} \qquad (12)$$

where X is the concentration of κ-casein in g/l; and C_0 and C_1 are soluble species of α_{s1}- and/or κ-casein. S_i or k_i is the parameter from Eq. (4) studied. These colloid stability profiles were analyzed as discussed above.

3. RESULTS

3.1. Calcium-Induced Solubility Profiles of α_{s1}-Caseins

Figure 1 shows the calcium-induced solubility profile of purified α_{s1}-casein *A*. Aliquot addition of Ca^{2+} results in a decrease in solubility resulting in precipitation at concentrations above 10 mM Ca^{2+}. In order to quantify the data, nonlinear regression analyses were performed. The data of Fig. 1a were fitted by Eq. (4) with k_2 and k_3 set at zero (reducing the data to a two-state model). Estimates of k_1, were obtained at fixed integer values of n ranging from 1–12; the final value of n was taken to be the fit with the minimum root mean square (RMS), the minimum estimated error of the coefficients, and no significant improvement in fit (Meites, 1979). Figure 1a shows the fit to $n=2$, 4, and 8 for α_{s1}-*A*. Values for $n=8$ gave the minimum RMS with the lowest error in k_1, values >8 caused the fit to diverge. The degree of divergence places an error of ±1 on n. In each case, however, a statistical minimum can be obtained. The

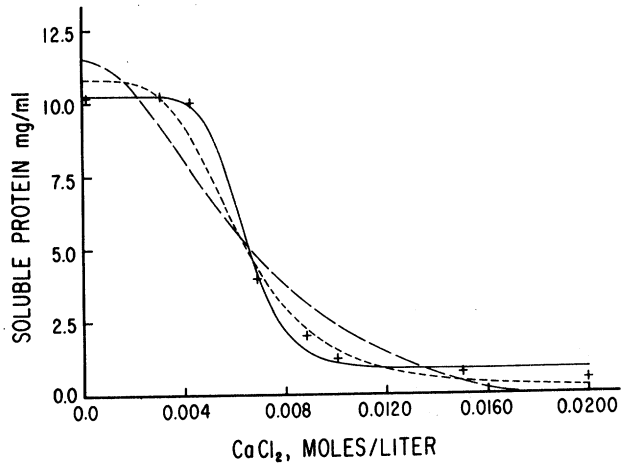

Fig. 1. Solubility at 37°C of the calcium salts of α_{s1}-casein *A* as a function of increasing $CaCl_2$ concentration. Solutions buffered at *p*H 7.0, 10 mM imidazole-HCl. The experimental data for α_{s1}-*A* with added 0.07 M KCl were fitted by Eq. (4) by nonlinear regression analysis with values of 2 (— — — —), 4 (- - -), and 8 (——) assigned to n. The best fit obtained for $n=8$, where the RMS and errors of the coefficients reached a minimum. RMSs for $n=2$, 4, 6, 8, 10, and 12 were: 1.236, 0.533, 0.233, 0.230, 0.294, and 0.337, respectively; the errors of coefficients were 28%, 7.0%, 2.4%, 1.9%, 2.0% and 2.1%, respectively.

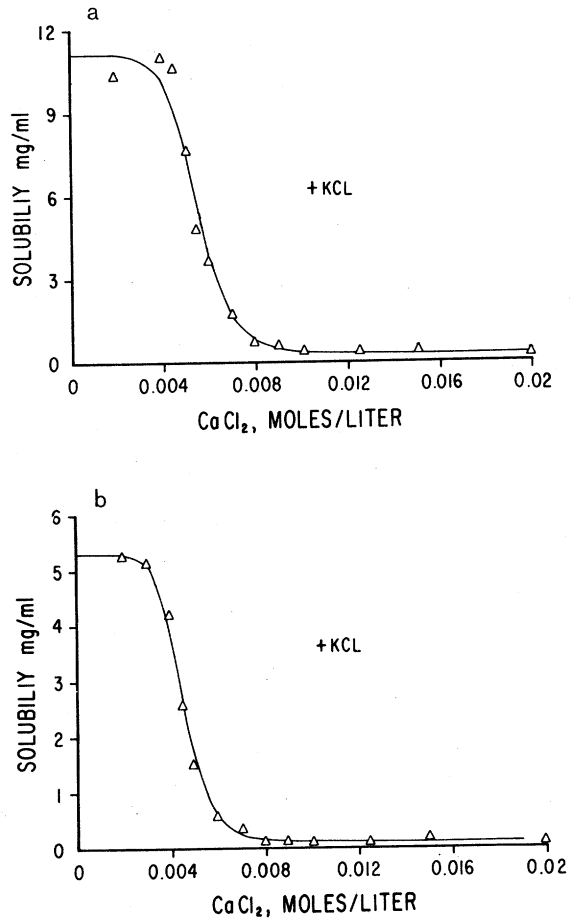

Fig. 2. Solubility at 37°C of calcium α_{s1}-*B* caseinates as a function of increasing $CaCl_2$ in KCl at two different concentrations: (a) 10 mg/ml; (b) 5.5 mg/ml. Data were fitted with Eq. (4); results are given in Table 1.

Table I. Comparison of Calcium-Induced Solubility of α_{s1}-Caseins *A* and *B* at 37°: Effects of Salt and Ca^{2+} States

Protein concentrations[a]	k_1-Ca^{2+}_{Total}[b]	k_1-Ca^{2+}_{free}	S_1[c]	S_0[c]	*n*
α_{s1}-*A* (10+KCl)	157±3	236±2	10.1	0.9±0.2	8
(4−KCl)	180±4	214±3	4.1	0.6±0.3	8
α_{s1}-*B* (10+KCl)	186±3	296±2	10.2	0.2±0.1	8
(5+KCl)	225±2	270±5	5.3	0.1±0.1	8
(10−KCl)	151±1	240±4	9.4	0.5±0.1	16
(4−KCl)	204±3	245±3	4.0	0.1±0.1	16

[a] Protein initial concentrations in mg/ml±0.07 M KCl; experiments in 0.01 M imidazole buffer *p*H 6.7.
[b] *k*'s are expressed in L/mol.
[c] Solubility is in mg/ml.

computed value of k_1 was found to be 157 L · mol^{-1}. In a sense this represents the $[Ca^{2+}]_{Total}$ at which half of the protein is soluble, but it can also be an indication of K_a of binding for a class of sites which initiate precipitation of the kinetically active octomer described by Dalgleish *et al.* (1981). In the latter case, then, the *x* axis should represent $[Ca^{2+}]_{free}$. When a correction for the binding of Ca^{2+} to α_{s1}-casein is included as described in Experimental Procedures, then the data expressed in terms of $[Ca^{2+}]_{free}$ can be replotted and fitted again with Eq. (4). The analysis in terms of free or total Ca^{2+} are compared in Table 1. Values of S_0, S_1, and *n* did not change upon refitting of the data, only the k_1 values changed.

When data for two genetic variants (*A* and *B*) in the absence of 0.07 M KCl were fitted to the salting-out portion of Eq. (4) (terms 1 and 2) salting-out constants, (k_1-Ca^{2+}_{Total}) of 180 and 204 L · mol^{-1}, respectively, at 4 mg/ml protein were obtained. While these values are significantly different, more surprisingly, the number of apparent calcium binding sites needed to induce precipitation (*n*) differed as well (8 and 16, respectively). This latter difference was of interest, since in the presence of KCl (Farrell *et al.*, 1988) these values were equal to 8 for both variants. Effects of KCl on the precipitation of α_{s1}-*B* were tested (Fig. 2) at two different protein concentrations; data from Figs. 1 and 2 are summarized in Table I calculated for both total and free Ca^{2+}. With no KCl present, k_1 is significantly smaller and *n* is 16 for both protein concentrations. Conversely, in the presence of 0.07 M KCl, *n* is reduced to 8 for both concentrations of α_{s1}-*B*, and k_1 values are larger. If indeed these parameters are correlated with salt binding, then KCl, the predominant salt in milk serum (Farrell and Thompson, 1988), can be expected to have a significant influence on both the magnitude of k_1 and apparently the number of sites *n*. These effects could be correlated with a class of binding sites which are linked to changes in solubility of the α_{s1}-*B* genetic variant, but not the α_{s1}-*A*. The α_{s1}-*A* genetic variant is the result of the deletion of 13 amino acid residues from α_{s1}-*B* (Fig. 3). The loss of glu [14], glu [18], and arg [22], which are surrounded by hydrophobic side chains, may cause different physical properties for this protein; deletion of this peculiar segment may lead to altered equilibria among species present, particularly in the absence of competing salt, such as KCl. Indeed α_{s1}-*A* at 1°C is readily salted-in, whereas the *B* variant is not (Farrell *et al.*, 1988). If the individual protein exhibits different physical properties, it would be of interest to examine the effects of this deletion on the properties of the colloidal casein system.

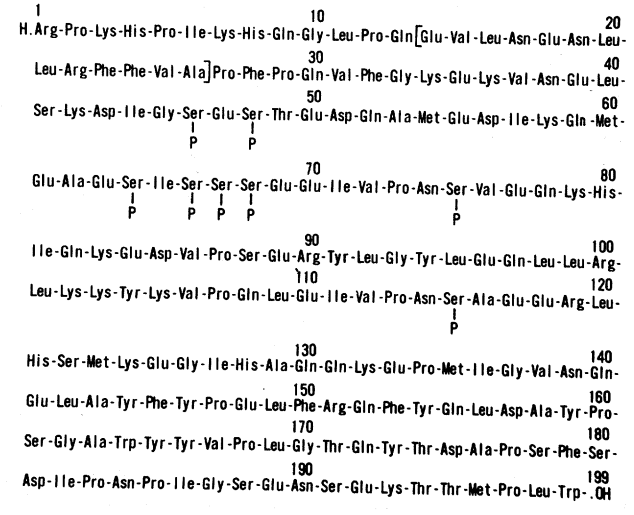

Fig. 3. Sequence of α_{s1}-casein *B*, showing amino acids deleted to yield the α_{s1}-*A* variant.

3.2. Sedimentation Velocity and Colloidal Stability

As noted above the Ca^{2+} insoluble α_{s1}-caseins are stabilized by the Ca^{2+}-soluble κ-casein to form colloidal complexes. These complexes, which have approximate radii of 650 Å, are stable to 1 *g*. Previous researchers (Noble and Waugh, 1975; Thompson *et al.*, 1969) have defined the colloidal stability of casein micelles as that fraction soluble after sedimentation at 1500 *g* for 10 min. To test the validity of this method, analytical ultracentrifugation of model micelles was carried out. Figure 4a shows a typical sedimentation velocity profile of model micelles of whole α_{s1}-*B* caseinate at 847 *g* and 10 min of sedimentation. Using nonlinear regression analysis, each scan was fitted by an integral of a gaussian to determine the position of the boundary. Several inferences can be made by examination of Fig. 4. In these experiments, no solid material is seen at the bottom of the cell, indicating that the particles are stable and are not rapidly sedimented under these conditions. Second, fits to integrals of gaussians were quite good with an average degree of confidence of 90–95%, implying relatively good homogeneity of the particles. To test the fits, deviation analysis was carried out as suggested by Meites (1979). A pseudo-random pattern for the deviation was obtained as shown in the inset of Fig. 4. However, plots of the spreading factor vs. time (not shown) gave nonlinear data indicating, as shown by electron microscopy (Schmidt 1984), that some polydispersity does occur among these model complexes, although on the whole, the model system possesses a characteristic size distribution. For the purpose of developing a test for colloidal stability the data seem adequate and the conditions empirically chosen by previous investigators (1500 *g* for 10 min) appropriate.

The average sedimentation constant (2090 *S*) obtained for these model micelles can be used along with Svedberg's equation to calculate both the molecular weight and the Stokes radius of the micelles, assuming hydrations obtained from previous

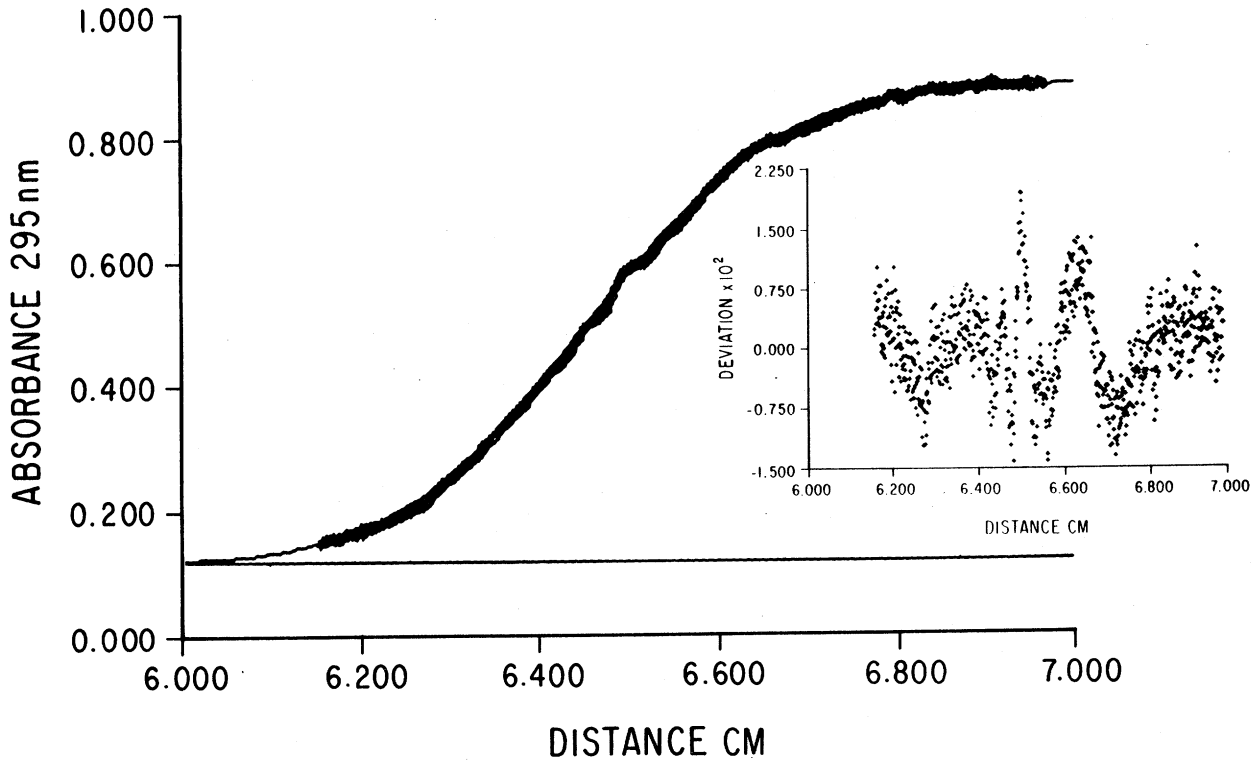

Fig. 4. Sedimentation velocity profile of model α_{s1}-*B* micelles; sodium caseinate typed α_{s1}-*B* and containing 12% κ-casein was dissolved in 0.01 M imidazole-HCl buffer containing 0.07 M KCl at 2 mg/ml. Buffered $CaCl_2$ was added to make the final concentration of protein 0.5 mg/ml and the final $CaCl_2$ 10 mM. Sedimentation velocity was carried out at 847 *g*. A wavelength of 295 nm was used to measure the progress of the boundary; spectrophotometric assays showed that at this wavelength Beer's law is obeyed between 0.1 and 1.0 mg/ml protein. Inset shows the deviation pattern of the same fitted curve as determined by the method of Mieties (1979).

Table II. Comparison of Molecular Parameters of Model Casein Micelles Calculated from Sedimentation Velocity, with Literature Data

Source	$S_{20,w}$	Molecular weight and method[a]	r, Å and Method[a]
This study[b,c]	2090	600×10^6	665 (UC)
Holt *et al.*	2800	1700×10^6 (LS)	1500 (LS)
Linn *et al.*	—	85×10^6 (DLS)	650 (DLS)
Schmidt *et al.*	—	—	674 (EM)

[a] Methods of measurement: UC = velocity sedimentation; EM = medium volume radius electron microscopy; LS = conventional scattering; DLS = dynamic light scattering.

[b] Initial concentration 1 g/L at 20°C and 20 mM $CaCl_2$; average value from five experiments, standard error of the mean was 8%.

[c] The molecular weights and radii were calculated using $\bar{v} = 0.736$, from Kumosinski *et al.* (1987), and assuming an average hydration of 0.5 g H_2O/g protein.

NMR data (Kumosinski *et al.*, 1987). The calculated values are in reasonable agreement (Table II) with data from light scattering and sedimentation studies (Holt *et al.*, 1987; and Linn *et al.*, 1971) and electron microscopy (Schmidt *et al.*, 1973). Thus, colloidal stability can be defined as the fraction which does not sediment at 1500 *g*, while colloidal instability (coagulation) is defined as the fraction sedimented under these conditions. As an extension of the previously developed theories on solubility (Farrell *et al.*, 1988), k_1 may still be thought of as calcium-binding linked to precipitation (salting-out), while k_2 may be more appropriately defined as a colloidal stability constant (salting-in), whose linkage is perhaps affected by casein–casein interactions. Moreover, instability of the system as a whole can be measured through the linkage of k_3 with calcium binding (colloidal destabilization). In this light, the S_n terms represent concentrations of stable colloid.

3.3. Model Casein Colloids

Colloidal stability tests, as defined above, were carried out on model micelles made from mixtures of the purified α_{s1}-caseins *A* and *B* and κ-casein (Fig. 5a and b). While the α_{s1}-*A* micelles (Fig. 5a) are soluble at total Ca^{2+} concentrations of 16 mM, α_{s1}-*B* micelles are less soluble at these concentrations (Fig. 5*b*). In the case of α_{s1}-*B*, κ-casein initially enhances stability at lower Ca^{2+} concentrations, but

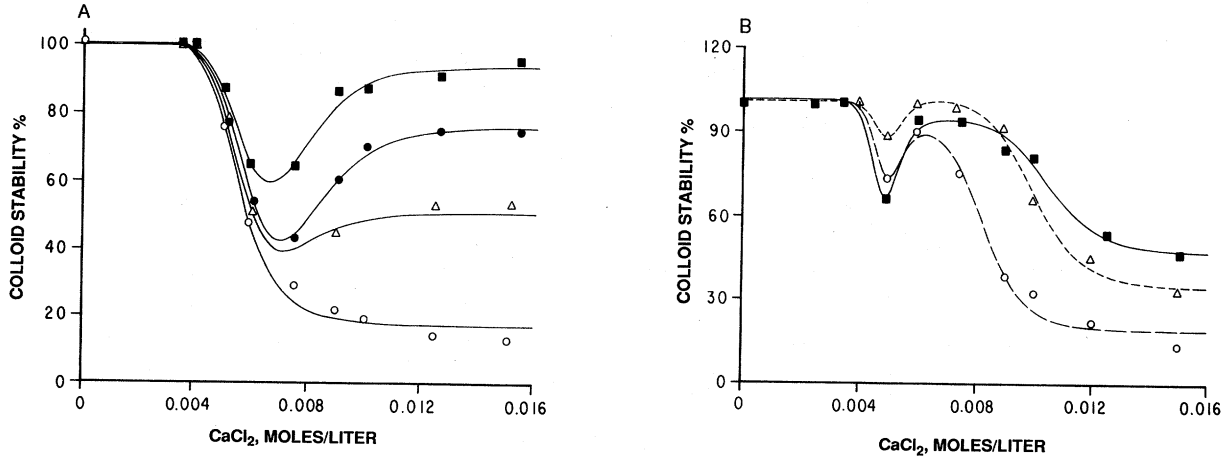

Fig. 5. Colloidal stabilities for reformed micelles. (A) Supernatant protein at 37°C resulting from the incremental addition of $CaCl_2$ to α_{s1}-*A*, no κ-casein(○); α_{s1}-*A* + κ-casein, 40:1 (△); α_{s1}-*A* + κ-casein, 20:1 (●); and α_{s1}-*A* + κ-casein, 10:1 (■). Solutions buffered at *p*H 7.0, 0.01 M imidazole-HCl. Initial protein = 4 mg/ml. Data were fitted with Eq. (4); results are given in Table III. (B) Supernatant protein at 37°C resulting from the incremental addition of $CaCl_2$ to α_{s1}-*B* + κ-casein, 40:1 (○); α_{s1}-*B* + κ-casein, 20:1 (△); and α_{s1}-*B* + κ-casein, 17:1 (■). Solutions buffered at *p*H 7.0, 0.01 m imidazole-HCl. Initial protein = 4 mg/ml. Data were fitted with Eq. (4); results are given in Table IV.

Table III. Effect of κ-Casein on Calcium-Induced Insolubility of α_{s1}-Casein *A* at 37°C

Ratio[a]	k_1[b]	k_2	S_1, %[c]	S_2, %[d]
No-κ-	180±3 (214)	—	17±2	—
40:1	174±4 (200)	138±4 (160)	1.6±0.9	51±3
20:1	170±2 (201)	127±2 (147)	1.5±0.9	77±1
10:1	169±3 (200)	137±2 (161)	1.4±0.9	95±2

[a] Ratio of α_{s1}-*A* to κ-casein. Initial concentration 4 mg/ml α_{s1}-*A*.
[b] $n = m = 8$ for all calculations; k is expressed as L/mol; numbers in parentheses represent k_i-Ca^{2+}_{free}; the others are for Ca^{2+}_{Total}.
[c] S_1, percentage of total protein soluble after initial precipitation.
[d] S_2, percentage of resoluble casein at elevated calcium concentration.

Table IV. Effect of κ-Casein on Calcium-Induced Insolubility of α_{s1}-Casein *B* at 37°C

Ratio[a]	k_1[b]	k_2	k_3	S_3, %[c]
No-κ-	204±10	—	—	—
40:1	211±21	197±22	121±4 (140)	20±3
20:1	206±21	198±20	100±2 (115)	35±3
17:1	214±11	189±10	95±3 (109)	36±3
7:1	210±10	190±20	62±2	40±4

[a] Ratio of α_{s1}-*B* to κ-casein. Initial concentration 4 mg/ml α_{s1}-*B* (S_0).
[b] $n = m = 16$ for all calculations, and $q = 12$ k's are expressed L/mol. Numbers in parentheses represent k_3-Ca^{2+}_{free}; all others are for Ca^{2+}_{Total}.
[c] All S_2 values at 100%; S_1 at 0; S_3 represents % α_{s1}-*B* soluble at elevated calcium concentrations.

increased Ca^{2+} leads to precipitation. For α_{s1}-*A*, increased Ca^{2+} does not lead to decreased stability. The data for α_{s1}-*A* were analyzed by use of terms 1 and 2 of Eq. (4), with $k_3 = 0$; results are summarized in Table III for both Ca^{2+}_{Total} and Ca^{2+}_{free} concentrations. As κ-casein is added to α_{s1}-casein, increased stability occurs across the profiles. The salting-out constant (k_1) is not appreciably different for α_{s1}-A as κ-casein is varied, but salting-in (k_2) is lower than k_1 and, although it does not vary with κ-casein concentration, its value is 10-fold greater than salting-in (k_2) for α_{s1}-*A* in the absence of κ-casein (Farrell *et al.*, 1988). Thus, κ-casein facilitates colloidal stability.

In the case of α_{s1}-*A* the amount of soluble colloid (S_2) depends upon κ-casein content. The variation of S_2 with κ-concentration is plotted for α_{s1}-*A* (Fig. 6). Treating these data as a binding isotherm [Eq. (12)] for the interactions of α_{s1}- and κ-, a value of $n = 1$ was found for α_{s1}-*A* from the variance of S_2 with κ-casein, and an association constant for the α_{s1}-*A*-κ-complex could be calculated. This value (10 L g^{-1}) is in good agreement with literature data for these associations (Schmidt, 1984).

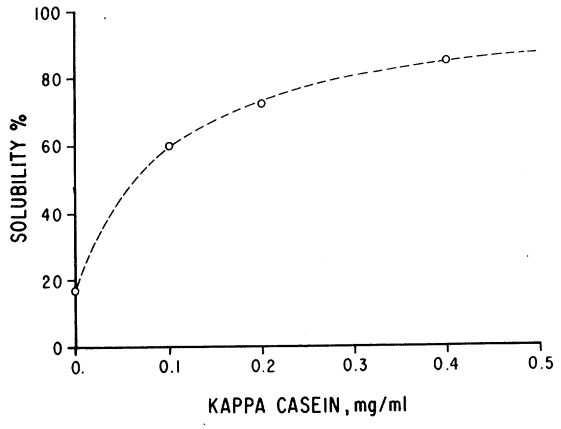

Fig. 6. Variation of S_2 (α_{s1}-*A*) with added κ-casein (○). Data were analyzed as with Eq. (12).

The α_{s1}-*B* variant, as noted above, shows significant destabilization of the colloidal complexes as the Ca^{2+} concentration exceeds 10 mM, even at elevated κ-casein contents. In order to analyze these data, the parameters k^q and X^q were used. The results of the nonlinear regression analysis are given in Table IV, here only $[Ca^{2+}]_{Total}$ is considered for k_1 and k_2. Again, k_1 does not vary with κ-casein and is not different from the salting-out constant obtained in the absence of this stabilizing protein (κ-casein). Like α_{s1}-*A*, the k_2 for the *B* variant is significantly increased over values found in the absence of κ-casein at 1°C; in fact, this variant is usually nearly completely insoluble without κ-casein. The amount of soluble protein (S_3) here represents that which remains after colloid formation and subsequent destabilization. This parameter varies with κ-casein content, and q has an unusually large value of 12.

3.4. Variation of Derived Parameters for the Interaction of α_{s1}-*B* and κ-Caseins

The variation of k_3 (destabilization of the micelles) for α_{s1}-*B*-κ-casein complexes as a function of κ-casein content is shown in Fig. 7. As the ratio of α_{s1}- to κ- decreases, k_3 (from Ca^{2+}_{Total} or Ca^{2+}_{free})

Table VI. Comparison of Association Constants for Various Calcium Complexes with Values Obtained for k_1

Complex of Ca^{2+}	log K_a [a]	log k_1-Ca^{2+}_{Total} [b]	log k_1-Ca^{2+}_{free} [b]
α_{s1}-Casein *B*	2.6	2.31 ± 0.04[c]	2.39 ± 0.04[c]
α_{s1}-Casein *A*	—	2.26 ± 0.06[c]	2.33 ± 0.06[c]
α_{s1}-*B* colloid stability	—	2.32 ± 0.04[d]	—
α_{s1}-*A* colloid stability	—	2.23 ± 0.05[e]	2.30 ± 0.05[e]
β-Casein	2.6		
O-Phosphoserine	2.2		
Glutamate	0.8		
Acetate	0.6		

[a] From the data of Dickson and Perkins (1971).
[b] Errors for log *k*'s estimated from $\Delta\log = 2.303 \times \Delta k/k$.
[c] From Table 1, 4 mg/ml no KCl.
[d] From Table IV, average values k_1.
[e] From Table III, average values k_1.

4.2. Calcium-Induced Solubility Changes

The calcium-induced precipitation of the isolated α_{s1}-*A* and *B* yielded lower values of k_1, in buffer alone as compared to 0.07 M KCl; also *n* was twofold larger for α_{s1}-*B* (16 vs. 8). The effect of salt on these parameters was studied. If k_1 is related to calcium binding, then with no KCl present weaker sites can interact, and k_1 decreases for α_{s1}-*B* regardless of protein concentration (Table I). In the case of *n*, the values obtained in KCl (8 for the α_{s1}-caseins) appear to correspond to the number of moles of calcium bound as calculated from the data of Dickson and Perkins (1971) carried out in 0.1 M NaCl. Coincidentally, these values also correspond to the number of phosphate residues found by sequence for the respective caseinates (Eigel *et al.* 1984). α_{s1}-Casein *A* contains a total of 29 carboxylate and 8 phosphate groups. The net charge on this latter protein at neutral *p*H, can be calculated to be −24; theoretically, binding of eight divalent calcium ions would decrease this number to −8, but the K_a for calcium binding is quite low (Table VI), and so site occupancy is not high. Using the published K_a values, protein concentration and Ca^{2+}_{Total}, free and bound Ca^{2+} may be estimated. In general, when binding is taken into account pk_i values for the precipation reaction are not dramatically altered (Tables I and VI). The binding "seen" by linked function analysis, while related to the net binding, is actually more related to the physical property studied. Under the conditions in which Ca^{2+} is bound but no precipitation occurs (Fig. 1 concentrations up to 4 mM), α_{s1}-*B* polymerizes to an octamer, the proposed kinetic unit in the precipitation reaction. Only that binding directly linked to observed solubility changes is disclosed by the linked function analysis; this occurs at higher Ca^{2+}_{Total} concentrations. Initially, the values of k_1 and *n* calculated for α_{s1}-*A* and *B* in 0.07 M KCl seemed to agree. In the absence of KCl they diverge. These observations are not contradictory, if altering the protein (genetic variation in this instance) leads to altered solubility, then the equations disclose those binding sites linked to this phenomenon, under the specific conditions used. Indeed using isolated α_{s1}-casein *B*, Parker and Dalgleish (1981) have shown that NaCl can alter both *n* and K_a in equilibrium dialysis studies. The major implication of the present work is that these interactions described for the individual casein components (Farrell *et al.*, 1988), may carry over into the colloidal complexes themselves.

4.3. Calcium-Induced Colloidal Stability

Model colloidal complexes were previously defined by their stability at 1500 *g* (Noble and Waugh, 1965; Thompson *et al.*, 1969). Analytical ultracentrifugation of dilute solutions showed that under these selected conditions, the molecular weights and sizes of the calcium-induced soluble complexes are indeed in the order expected for casein micelles (Table II). The effects of the genetic variants *A* and *B* were tested in this system. Although the conditions for centrifugation are altered from the solubility studies described above, an initial decrease in solubility does occur, but this is followed by a "salting in" or colloidal stability reaction. Figure 5a and b show the stabilization of α_{s1}-*A* and *B* by κ-casein (*p*H 7.0 imidazole-

HCl buffer, 37°C) as a function of increasing calcium ion concentration. While these experiments were performed in the absence of KCl, the dip at 5 mM $CaCl_2$ reported by Noble and Waugh (1965) was apparent. In their early experiments on the colloidal system, these latter workers established that under these conditions, the various soluble species of protein present (which are, of course, produced by calcium binding) are in thermodynamic equilibrium. These conclusions were later confirmed by Dalgleish *et al.* (1981). Thus, Eq. (1), cast in the form of a binding isotherm, relates the proposed stoichiometric terms (p, PX_m, etc.) to the fraction present in these soluble species (S_0, S_1, etc.). Analysis of this data for α_{s1}-*B* complexes with Eq. (4) showed no substantial changes in k_1 from those found at 37°C with no κ-casein present (Table VI), even though the centrifugation conditions are altered. On the other hand, κ-casein caused a 10-fold increase in k_2, over the salting-in constants, found for salt alone at 1°C for the α_{s1}-caseins (Farrell *et al.*, 1988). In the absence of κ-casein at 37°C, the α_{s1}-*A* and *B* are nearly completely insoluble. κ-Casein could act in three ways to induce formation of stable colloidal complexes. In the first case, the κ- could simply bind calcium and thus prevent calcium precipitation; equilibrium dialysis experiments (Dickson and Perkins, 1971), however, show that κ-casein has a lower K_a for calcium than α_{s1}- and has only 1 to 2 binding sites under these conditions. A second alternative is that κ-casein interacts with the α_{s1}- in such a way as to totally prevent calcium binding; this can be argued against since k_1 and n are essentially independent of κ-casein concentration for both genetic variants (Table VI), and since mixtures of caseins do bind calcium (Dickson and Perkins, 1971). The third alternative is that the α_{s1}- and κ-casein form complexes with altered affinity for calcium which have the ability to go on to produce collodial particles. This latter alternative appears most likely. Formation of protein complexes in the absence of Ca^{2+} has been demonstrated by free boundary electrophoresis and analytical ultracentrifugation (Schmidt, 1984) and by gel permeation chromatography (Pepper and Farrell, 1981). As clearly shown in this work, by ultracentrifugation, calcium does generate complexes of colloidal dimensions (Table II) in our model systems. Considering α_{s1}-*B* in 0.07 M KCl as the starting point, both the addition of κ-casein or the omission of salt decrease k_1 but increase n from 8–16. This indicates either that additional weaker sites for calcium are exposed by such treatments or that the active kinetic unit in octamerization may be a "dimer." In either case, a structural change accompanying protein-protein interactions between κ- and α_{s1}- could occur in α_{s1}-*B*, perhaps preventing octamerization and formation of the species predominant in the precipitation reaction (Dalgleish *et al.*, 1981). Evidence for an increase in the number of β turns in mixtures of caseins (*B*-variant) over the sum of the individual proteins has recently been given by Raman and FTIR spectroscopy (Byler *et al.*, 1988; Byler and Farrell, 1989). This data supports the hypothesis that protein-protein interactions induce some structural changes in the caseins. κ-Casein thus interacts with α_{s1}- and facilitates colloidal stability.

In these studies k_2 is increased nearly 10-fold by κ-casein over the value for salting-in of the α_{s1}-caseins alone; k_2 may thus be considered a measure of colloidal stability rather than salting-in. A monotonic increase in total amount of soluble protein was observed for α_{s1}-*A* (Fig. 5a) with increasing κ-casein; these data are quantitated in Table III. The variation of S_2 with κ-casein was subjected to nonlinear regression analysis by Eq. (4), and this yielded an equilibrium constant of 78,000 L mol^{-1} for the interaction of κ-casein with α_{s1}-*A* at 37°C. This value is in reasonable agreement with the value of 71,600 L mol^{-1} from the fluorescence polarization analyses of Clarke and Nakai (1971) at 35°C.

Comparison of Fig. 5a and b shows another important difference between α_{s1}-caseins *A* and *B*. As the Ca^{2+} concentration is increased, the colloidal complexes containing α_{s1}-*B* begin to destabilize and start coagulating; α_{s1}-*A* complexes do not. The data for α_{s1}-*B* were treated by an extension of the general salting-out theory (Farrell *et al.*, 1988). Addition of a third term for colloidal destabilization [Eq. (2)] yields k_3 and q, which can be given the same interpretations as k_1 and n, but are applied to the larger micellar complexes of the α_{s1}-κ-calcium caseinates. Here the κ-casein is able to protect α_{s1}-*B* from precipitation at lower Ca^{2+} concentrations, but as Ca^{2+} increases destabilization occurs. In contrast, α_{s1}-*A*-κ-casein complexes remain stable. The basis for this difference between the *A* and *B* variants may be as follows. α_{s1}-*A* represents a sequential deletion of 13 amino acid residues (Fig. 3). Note that residues 14 and 18 are glutamic acids, while residue 22 is an arginine, the remainder of the amino acid residues deleted are hydrophobic or noncharged. It is postulated that an intermolecular hydrophobically stabilized ion-pair (as described by Tanford, 1967) exits between α_{s1}-*B* monomers. Such a species might be

argued for on the basis of the two-fold change in n between the A and B forms of α_{s1}-casein. For α_{s1}-B, if dimerization occurs, then $n = 16$ would represent calcium binding to this "dimer." Resolubilization, or more appropriately, colloid stabilization, occurs with $m = 16$. The absence of this bond in α_{s1}-A results in more salt-stable colloidal complexes with lower n; or, viewed another way, α_{s1}-B and its complexes are more readily destabilized.

Figure 7 shows the effect of added κ-casein on the calcium-induced destabilization of model casein micelles of α_{s1}-B. As κ-casein content increases, k_3 (a measure of destabilization) decreases (Table IV). In fact, at abnormally elevated κ-casein to α_{s1}-casein ratios (Fig. 8 inset), complete stabilization is achieved. Since full stabilization is accomplished for α_{s1}-A at more normal κ-casein concentrations, we look to the genetic deletion as the source of the instability in the B variant. If added κ-casein is acting to stabilize the hypothetical ion-pair alluded to above in α_{s1}-B, then the change in ΔG destabilization by added κ-casein may be related to the energy of the bond stabilized. If we take extrapolated y-intercept of Fig. 7 (172 L/mol) as k_3^0 the constant for destabilization without κ-casein, then the increase in colloidal stabilization with increased κ-casein equivalent to normal milk ($k_3 = 62$ L/mol, Table IV) could give a measure of the colloidal stabilization energy resulting from added κ-casein:

$$\Delta G\text{-colloidal stabilization} = \Delta G(k_3^0) - \Delta G(k_3)$$
$$= -0.63 \text{ kcal/mol}$$

Note that an identical value is obtained if k_3 is recast in terms of Ca^{2+}_{free}. At first sight, this value appears rather small, but the same value of ΔG could predict a pK shift of 0.38 units for a protein carboxyl group. Moreover, when compared to the overall ΔG of stabilization of globular proteins, which averages ~8 kcal/mol (Privalov, 1979), this value could be a significant portion of casein energy of stabilization, especially when the "open" structure of casein is considered. While this does not prove the existence of an intermolecular hydrophobically stabilized ion-pair, it does suggest that the deleted segment plays a major role in casein interactions in normal milks.

From all of the above, it can be seen that genetic alteration of protein interaction sites can produce major changes in the physical properties of the protein. Since α_{s1}-B is the most abundant genetic variant of the caseins, its more conventional properties may have been "selected for" in conventional breeding. That being the case, the postulated ion pair may be important in coagulation, but its absence could lead to new products. Richardson and co-workers have recently cloned the α_{s1}-A gene, which could lead to further site-specific changes in these proteins (McKnight *et al.*, 1987).

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